A proteomics approach to inner membrane biogenesis in Escherichia coli

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Chapter 3

Differential effect of YidC depletion on the membrane proteome of *Escherichia coli* under aerobic and anaerobic growth conditions

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YidC of *Escherichia coli* belongs to the evolutionarily conserved Oxa1/Alb3/YidC family. Members of the family have all been implicated in membrane protein biogenesis of respiratory and energy transducing proteins. However, the number of proteins identified thus far to require YidC for their membrane biogenesis remains limited. The identification of new YidC-dependent proteins may allow the elucidation of properties that confer YidC-dependence to this subset of membrane proteins. To this end we investigated changes in the membrane proteome of *E. coli* upon YidC depletion using metabolic labeling of proteins with $^{15}N/^{14}N$ combined with a mass spectrometry-centred proteomics approach. It has been shown previously that YidC is essential for growth under both aerobic and anaerobic growth conditions and we therefore compared the effects of YidC depletion under both growth conditions. We found that YidC depletion resulted in protein aggregation/misfolding in the cytoplasm as well as misfolding in the inner membrane of *E. coli.* A dramatic increase was observed in the chaperone-mediated stress response upon YidC depletion and interestingly, this response was limited to the aerobically grown cells. A number of inner membrane transporter proteins were identified as possible candidates for the YidC-dependent insertion and/or folding pathway. These included the small metal ion transporter CorA, the ABC arginine, dipeptide, histidine, branched amino acid, lipoprotein-releasing, lipopolysaccharide and putrescine transporters, as well as the MFS transporters KgtP and ProP, providing a new subset of proteins potentially requiring YidC for membrane biogenesis.

Manuscript in preparation

INTRODUCTION

Approximately 30% of proteins encoded by *Escherichia coli* are destined for insertion into or translocation across the inner membrane. Membrane proteins perform many of the cell's essential functions such as energy transduction, solute transport and stimulus transduction. The general secretory (Sec) pathway is responsible for insertion of the majority of integral membrane proteins. In this pathway, ribosome-bound nascent chains are targeted by the bacterial signal recognition particle (SRP) to the Sec translocase via the SRP receptor FtsY (For reviews see (124,282)). The protein conducting channel of the Sec translocase consists of the heterotrimer SecYEG (75,496). Associated with the channel is the motor protein SecA which drives the translocation of large polar intermembrane loops across the membrane (426). A heterotrimeric complex consisting of SecD, SecF and YajC is associated with the Sec translocase but the functions of these proteins is unclear (76,133,297). Another protein found associated with the Sec translocase, YidC, can function together with the SecYEG channel as well as independently as a membrane insertase for small hydrophobic proteins.

YidC belongs to the evolutionarily conserved Oxa1/Alb3/YidC family which evolved before the divergence of the 3 major domains of life (281,366,542). Oxa1 (Oxidase assembly) from yeast was the first member of this family to be described. It was originally identified as an essential factor for the biogenesis of respiratory complexes in the mitochondrion (31,64), more specifically the insertion of subunits of the cytochrome bc_1 oxidase and ATP synthase (15). Alb3 is located in the thylakoid membranes of *Arabidopsis* chloroplasts (319) and is involved in the biogenesis of light harvesting complexes. Members of the family have all been implicated in membrane protein biogenesis of respiratory and energy transducing proteins. To date, only two natural *E. coli* substrates have been identified as using the YidC-only, or Sec-independent, pathway for insertion, namely the c subunit of the F_1F_o ATP synthase, F_oc (499), and the channel of large conductance, MscL (145). The phage coat proteins M13 and Pf3 also make use of the YidC-only pathway for insertion (91,409). Subunit a of the F_1F_o ATP synthase, F_oa (544), and subunit II of the cytochrome *o* oxidase, CyoA (126,494,494), are inserted via the Sec pathway but YidC is also essential for their insertion. It has been proposed that for CyoA YidC inserts the first helical hairpin after which SecYEG inserts the C-terminus (87). YidC has also been implicated in the folding of proteins following their insertion by the Sec translocase. The lac permease, LacY (326), and MalF, of the maltose transport complex (514), have been shown to require YidC for correct folding and

stability in the membrane.

The number of proteins identified thus far to require YidC for their membrane biogenesis remains limited. In general, YidC and other Oxa1-related proteins appear to be involved in the integration of small hydrophobic proteins that form part of larger oligomeric complexes. The identification of new YidC-dependent proteins may allow the elucidation of properties that confer YidC-dependence to this subset of membrane proteins. To this end we investigated changes in the membrane proteome of *E. coli* upon YidC depletion. The proteomic analysis of membrane proteins poses a number of challenges. Owing to their hydrophobic nature, membrane proteins are not suited to standard two dimensional (2D) gel-based methods. 2D gel analysis in which the first dimension is replaced by separation by blue native PAGE (BN-PAGE) followed by standard SDS-PAGE helps eliminate this problem and allows comparative proteomic analysis (255). Metabolic labeling of proteins with stable isotopes offers an alternative quantitation method. This can be combined with MS-centred proteomics approaches based on the separation of complex trypsin-digested samples by reverse phase chromatography in combination with mass spectrometric analysis (LC-MS/MS). This offers a greater sensitivity than gel-based quantitation approaches (123,516,528).

It has been shown previously that YidC is essential for growth under both aerobic and anaerobic growth conditions (376,408) and we therefore compared the effects of YidC depletion on cells grown aerobically with that of those grown anaerobically with fumarate as terminal electron acceptor. YidC depletion under aerobic conditions elicited a massive chaperone response to protein misfolding and/or aggregation while depletion under anaerobic conditions did not show this response. A number of inner membrane transporter proteins were identified as possible candidates for the YidC-dependent insertion and/or folding pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids - The YidC depletion strain *E. coli* FTL10 (196) in which *yidC* is under the control of the *araBAD* promoter was a generous gift of Frank Sargent (University of East Anglia, Norwich, United Kingdom). *E. coli* FTL10 "YidC^{+}" and "YidC^{-}" strains were used as described (376). Plasmids pEH1YidC (500) and pTrcYidC (402) were used for the overexpression of His-tagged and non-tagged versions of YidC respectively.

Materials - Sodium fumarate was purchased from Sigma-Aldrich. ^{15}N-ammonium

sulfate was purchased from Cambridge Isotope Laboratories (USA) and trypsin from Promega (Germany). Antisera against DnaK, F_oc, GroEL, IbpA, NarGHI, NuoK, PspA were generous gifts from Axel Mogk (Universität Heidelberg, Germany), Gabriele Deckers-Hebestreit (Universität Osnabrück, Germany), Saskia van der Vies (Vrije Universiteit Amsterdam), Ewa Laskowska (University of Gdansk, Poland), Axel Magalon (LCB-IBSM CNRS, Marseille, France), Takao Yagi (The Scripps Research Institute, USA) and Jan Tommassen (Utrecht University, The Netherlands), respectively. Polyclonal SecA and YidC antibodies were from our laboratory collection. Alkaline phosphatase conjugated anti-chicken, anti-mouse and anti-rabbit IgG were purchased from Sigma-Aldrich.

Bacterial growth - For metabolic labeling experiments using $^{14}N/^{15}N$, $YidC^+$ and $YidC^-$ strains were grown aerobically or anaerobically at 37°C. For aerobic growth, cell were grown in M63 minimal medium (96). To deplete cells of YidC, *E. coli* FTL10 harbouring either plasmid pET589 ("$YidC^+$") or pTrc99A ("$YidC^-$") were grown overnight in M63 minimal medium containing 0.5% (w/v) arabinose. Cells were then harvested, washed in minimal medium and resuspended in minimal medium containing glucose 0.5% (w/v) and growth was continued to an OD_{600} of 0.6. This corresponds to late exponential phase. The cells were then diluted 2-fold with the same medium and the procedure was repeated until the cessation of the "$YidC^-$" strain (adapted from 339). For anaerobic growth, cells were grown in basal anaerobic growth medium (277). Cells were depleted of YidC essentially as described above except that instead of glucose, glycerol and sodium fumarate were added to 0.5 % (w/v) and 10 mM respectively. Each strain was grown in duplicate in medium containing ^{14}N ammonium sulfate as well as in medium supplemented with ^{15}N ammonium sulfate. Cells grown in the presence of ^{15}N ammonium sulfate were used as a reference pool as described (286). After cells were harvested, equivalent OD-units of bacteria grown in the presence of ^{15}N or ^{14}N were combined and stored at -80°C.

Sample preparation – Inner membrane vesicles (IMVs) were isolated as previously described (241). The IMVs were subjected to sodium carbonate extraction (163) to further enrich for membrane proteins, and to reduce the amount of nonspecifically associated cytosolic proteins.

Mass spectrometric analysis – Inner membrane fractions were subjected to the GeLC MS- workflow including 1D SDS Gel electrophoresis followed by tryptic digestion as described in (123). The resulting tryptic peptides were separated on a reversed phase chromatography column (Waters BEH 1.7 μm, 100-μm

i. D. x 100 mm, Waters Corporation, Milford, Mass., USA) operated on a nanoACQUITY-UPLC (Waters Corporation, Milford, Mass., USA). Peptides were concentrated followed by desalting on a trapping-column (Waters nanoACQUITY-UPLC column, Symmetry C18, 5 µm, 180 µm x 20 mm, Waters Corporation, Milford, Mass., USA) for 3 min at a flow rate of 1ml/ min with 99% buffer A (90% acetonitrile, 0.1% acetic acid). The peptides were eluted and separated with a non-linear 80-min gradient from 5–60 % ACN in 0.1 % acetic acid at a constant flow rate of 400 nl/min. MS/MS- data were acquired with the LTQ-Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany) equipped with a nanoelectrospray ion source. After a survey scan in the Orbitrap (r= 30,000) MS^2 data was recorded for the five most intensive precursor ions in the linear ion trap.

The *.dta files were extracted from *.raw files using BioworksBrowser 3.3.1 SP1 (Thermo Fisher Scientific) with no charge state deconvolution and deisotoping performed on the data. The *.dta files were searched with SEQUEST version v28 (rev.12) (Thermo Fisher Scientific) against a *Escherichia coli* target-decoy protein sequence database (complete proteome set of *E. coli* K12 extracted from UniProtKB/ Swiss-Prot (465) with a set of common laboratory contaminants) compiled using BioworksBrowser. The searches were implemented in two iterations: First, for the GeLCMS analyses the following search parameters were used: enzyme type, trypsin (KR); peptide tolerance, 10 ppm; tolerance for fragment ions, 1 amu; b- and y-ion series; methionine oxidation as a variable modification (15.99 Da); a maximum of three modifications per peptide was allowed. In the second iteration the mass shift of all amino acids completely labeled with ^{15}N Nitrogen was taken into account in the search parameters.

Resulting *.dta and *.out files were assembled and filtered using DTASelect (version 2.0.25) (parameters GeLCMS: -y 2 -c 2 -C 4 --here --decoy Reverse_ -p 2 -t 2 -u --MC 2 -i 0.3 --fp 0.005). Data was revised using an in-house written java-script to ensure that each protein hit relied on at least two different peptides as judged by amino acid sequence.

Peptide ratios of heavy and light peptides according to the identification lists were determined by CenSus (358) and exported (R^2 values bigger than 0.7 and only unique peptides; proteins failing to be relatively quantified were checked manually in the graphical user interface for on/off proteins) to Excel. Proteins relatively quantified with at least 2 peptides were taken into account for the subsequent analysis.

Isolation of protein aggregates - Protein aggregates were isolated from $YidC^+$ and

YidC- cells according to the method described in (473). The method makes use of the selective solubility of protein aggregates and membrane proteins in the detergent NP-40. The samples were separated by SDS-PAGE and stained with Bio-Safe Coomassie (Bio-Rad, USA). In-gel tryptic digestion was followed by peptide extraction and LC-MS/MS as described in (402). Peptide mixtures from in-gel trypsin digestions were diluted in 0.1% TFA and separated on a C18 capillary column (C18 PepMap 300, 75 μm x 150 mm, 3 μm particle size, LC-Packing) mounted on an Ultimate 3000 nanoflow liquid chromatography system (LC-Packing) prior to trapping on a pre-column (300 μm x 5 mm, C18 PepMap300). Peptide elution was carried out with a gradient from 3 to 50% acetonitrile in 0.05% TFA in 40 minutes, at a flow rate of 300 $nL \cdot min^{-1}$. Column effluent was mixed 1:4 v/v with a matrix solution of 2.3 mg/mL α-cyano-4-hydroxycinnamic acid (LaserBio Labs) and fractions of 12 seconds width were spotted on a blank MALDI target with a Probot system (LC Packing). Mass spectrometric analysis was performed using a MALDI-TOF/TOF 4800 Proteomics Analyzer instrument (Applied Biosystems, USA) in the *m/z* range 900-4000. Data acquisition was performed in positive ion mode. Peptides with signal-to-noise level above 100 were selected for MS/MS. Protein identification was performed with ProteinPilot™ 2.0 software using the Paragon™ Algorithm (Applied Biosystems/MDS Sciex), searching against the *E. coli* K12 UniProtKB/Swiss-Prot protein sequence database. Trypsin specificity and all default options were included in the search. The results were manually inspected and identifications were accepted based on the independent identification of at least 2 unique peptides each with confidence of identification probabilities higher than 95 %.

Identification of co-purifying proteins - For the overexpression of (His-tagged) YidC, Luria Bertani (LB) medium was used for aerobic growth and the anaerobic basal medium was supplemented with 0.1% yeast extract (Difco). His-tagged YidC overexpressed from plasmid pEH1YidC was purified on a Ni^{2+}-NTA column in order to identify co-purifying proteins. The samples were separated by SDS-PAGE and identified as described for protein aggregates.

Protein determination and western blotting - Protein concentrations were determined with the DC Protein assay (Bio-Rad) using bovine serum albumin (BSA) as a standard. SDS-PAGE and immunoblot analyses were carried out according to methods previously described (264,474). Blotting efficiency was checked by immunoblot analysis using antisera directed against LepB (William Wickner,

Dartmouth University, USA). Signal capture and quantification were performed using the FUJIFILM LAS-4000 luminescent image analyzer.

RESULTS

In total 359 and 379 proteins were identified and quantified from the membrane preparations of aerobically and anaerobically grown *E. coli* cells respectively. Each dataset contained two biological repeats for each strain. Integral membrane proteins and inner membrane associated proteins were well represented (Figure 1). In the aerobic dataset 119 integral membrane proteins were quantified and from the anaerobic dataset 99 with a total of 134 different proteins quantified.

Proteins involved in membrane biogenesis - YidC was identified and quantified in all samples analyzed and was reduced in aerobic and anaerobic samples by a $\log_2$ factor of -3.37 and -4.32 respectively (Table 1). This corresponds to a 10 to 20-fold reduction in YidC indicating that YidC was extensively depleted during growth in the absence of arabinose. With the exception of the motor protein SecA, which increased upon YidC depletion under aerobic growth conditions, none of the other components of the general secretory pathway changed significantly upon YidC depletion under either aerobic or anaerobic growth conditions. A significant change in protein levels was taken to be 1.5-fold. Since SecA is localized in the

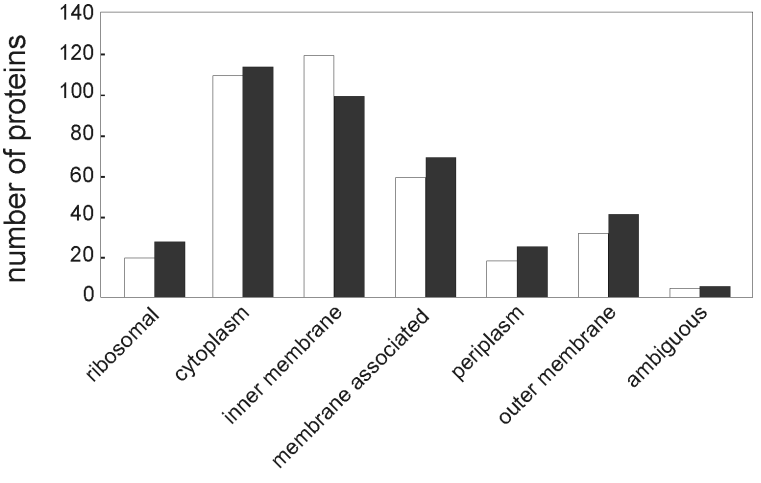

FIGURE 1. **Summary of localization of proteins quantified in this study**. Localizations were based on annotations in the UniProt KB/Swiss-Prot database for *E. coli* or where necessary Psort 2.0b (167). The number of proteins present in the aerobic (white bars) and anaerobic datasets (black bars) are presented.

TABLE 1. Proteins involved in membrane biogenesis

Gene name[a]	Protein name[b]	TMS / localization[c]	Log_2 ($YidC^+$-$YidC^-$)[d] Aerobic	Anaerobic
yidC (oxaA)	Inner membrane protein OxaA	6	**-3.37**	**-4.32**
ffh	Signal recognition particle protein	c	not identified	0.25
ftsY	Cell division protein FtsY	c, im ass	0.35	0.11
secA	Protein translocase subunit SecA	c, im ass	**0.68**	-0.09
secD	Protein-export membrane protein SecD	6	0.43	0.01
secF	Protein-export membrane protein SecF	6	0.13	0.14
secG	Protein-export membrane protein SecG	2	-0.34	-0.56
secY	Protein-export membrane protein SecY	10	-0.57	-0.50
yajC	UPF0092 membrane protein YajC	1	not quantified[e]	0.04
tatA	Sec-independent protein translocase protein TatA	1	0.08	0.26
tatB	Sec-independent protein translocase protein TatB	1	-0.57	-0.19
tatE	Sec-independent protein translocase protein TatE	1	not quantified	-0.17
tig	Trigger factor	c / im ass	not identified	-0.34

[a] Gene designations in the UniProtKB/Swiss-Prot database for *E. coli.*

[b] Protein designations in the UniProtKB/Swiss-Prot database for *E. coli.*

[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; im ass, inner membrane associated.

[d] The log_2 ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains.

[e] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.

Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

cytoplasm as well as associated with the inner membrane, the levels of SecA in cytoplasmic and membrane fractions were investigated using immunoblot analysis (Figure 2). Under aerobic growth conditions, the level of SecA in the cytoplasm did not change significantly upon YidC depletion, while the amount associated with the membrane increased. Under anaerobic conditions no change in the levels of SecA in the cytoplasm or associated with the membrane were observed in the immunoblot analysis. Under aerobic growth conditions, the levels of SecY decreased while under anaerobic conditions they decrease to a lesser extent. SecG and trigger factor, which contacts the nascent chain, decreased slightly upon YidC depletion while subunits of the heterotrimeric complex SecDFYajC, the SRP and its receptor, FtsY, increased slightly upon YidC depletion. Protein TatA, TatB and TatE which constitute the Sec-independent Tat translocase were also quantified and no significant changes were observed upon YidC depletion. Levels of TatB and TatE decreased while TatA was not negatively affected by YidC depletion.

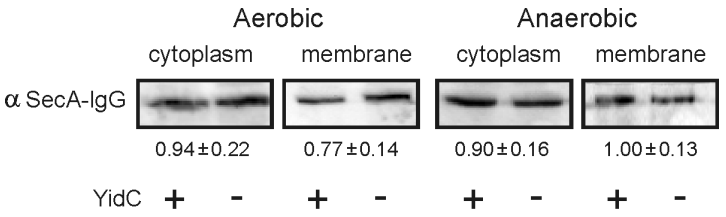

FIGURE 2. **An increased amount of SecA is localized to the inner membrane under aerobic growth conditions**. Cytoplasmic and membrane fractions were analyzed by immunoblotting using antisera directed against SecA. The ratio of the $YidC^+/YidC^-$ signals was calculated. Indicated are the means of three separate measurements ± the standard error of the mean.

Energy transducing proteins - Subunits of 14 different energy transducing complexes were quantified in the aerobic and anaerobic datasets. With the exception of FrdA of the fumarate reductase complex, and GlpB of the anaerobic glycerol-3-phosphate dehydrogenase, no significant changes were observed upon YidC depletion (Table 2). Six of the 13 subunits of the F_1F_o ATP synthase were quantified and although the levels of all the subunits were observed to decrease upon YidC depletion the changes were small. The YidC-dependent proteins F_oa and F_oc were not identified and the levels of F_oc in the membrane were therefore investigated by immunoblotting (Figure 3, upper panel). Under both aerobic and anaerobic

TABLE 2. Energy transducing proteins

Gene name[a]	Protein name[b]	TMS / localization[c]	Log_2 ($YidC^+$-$YidC^-$)[d]	
			Aerobic	Anaerobic
atpA	ATP synthase alpha subunit	c, im ass	-0.35	-0.08
atpC	ATP synthase epsilon subunit	c, im ass	not quantified[e]	-0.16
atpD	ATP synthase beta subunit	c, im ass	-0.36	-0.14
atpF	ATP synthase subunit b	1	-0.26	-0.12
atpG	ATP synthase gamma subunit	c, im ass	-0.37	-0.18
atpH	ATP synthase delta subunit	c, im ass	-0.49	-0.17
cydA	Cytochrome d ubiquinol oxidase subunit 1	7	-0.55	-0.11
cydB	Cytochrome d ubiquinol oxidase subunit 2	8	-0.07	0.12
cyoA	Ubiquinol oxidase subunit 2	2	-0.36	-0.36
cyoB	Ubiquinol oxidase subunit 1	15	0.02	-0.35
dmsA	DMSO reductase chain A	c, im ass	not identified	-0.19
dmsB	DMSO reductase chain B	c, im ass	not identified	-0.30
fdoG	Formate dehydrogenase-O major subunit	p, im ass	0.23	not identified
frdA	Fumarate reductase flavoprotein subunit	c, im ass	**-0.67**	0.00
frdB	Fumarate reductase Fe-S subunit	c, im ass	not quantified	0.03
frdC	Fumarate reductase subunit C	3	not identified	-0.06
frdD	Fumarate reductase subunit D	3	not identified	0.33
glpA	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	c, im ass	not identified	0.04
glpB	Anaerobic glycerol-3-phosphate dehydrogenase subunit B	c, im ass	not identified	**0.66**
glpC	Anaerobic glycerol-3-phosphate dehydrogenase subunit C	c, im ass	0.54	0.20
hybA	Hydrogenase-2 operon protein	p, im ass	not identified	0.32
hybC	Hydrogenase-2, large chain	p, im ass	not identified	0.17
narG	Respiratory nitrate reductase 1 alpha subunit	c, im ass	not identified	0.27
ndh	NADH dehydrogenase	c, im ass	-0.25	0.28

nuoA	NADH-quinone oxidoreductase subunit A	3	-0.58	-0.29
nuoB	NADH-quinone oxidoreductase subunit B	c, im ass	-0.16	-0.09
nuoC	NADH-quinone oxidoreductase subunit C/D	c, im ass	-0.18	-0.15
nuoF	NADH-quinone oxidoreductase subunit F	c, im ass	-0.02	not quantified
nuoG	NADH-quinone oxidoreductase subunit G	c, im ass	-0.11	-0.03
nuoI	NADH-quinone oxidoreductase subunit I	c, im ass	not quantified	0.20
nuoL	NADH-quinone oxidoreductase subunit L	14	-0.58	not identified
rnfG	Electron transport complex protein rnfG	1	-0.38	not identified
sdhA	Succinate dehydrogenase flavoprotein subunit	c, im ass	0.14	0.01
sdhB	Succinate dehydrogenase Fe-S subunit	c, im ass	-0.02	-0.03
ynfE	Putative DMSO reductase chain YnfE	c, im ass	not identified	-0.24
ynfG	Probable anaerobic DMS reductase YnfG	c, im ass	not identified	-0.40

[a] Gene designations in the UniProtKB/Swiss-Prot database for *E. coli.*

[b] Protein designations in the UniProtKB/Swiss-Prot database for *E. coli.*

[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; p, periplasm; im ass, inner membrane associated.

[d] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains.

[e] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.

Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

conditions, the levels of F_oc in the membrane decreased by approximately 2-fold consistent with the YidC-dependence of this protein for insertion.

Subunits of two cytochrome oxidases were quantified and the level of the previously characterized YidC-dependent CyoA decreased to the same degree upon YidC depletion under both conditions tested. CyoB levels did not change under either aerobic or anaerobic growth conditions. Under aerobic growth conditions the levels of CydA decreased significantly while the decrease in the anaerobic samples was less pronounced upon YidC depletion. CydB levels remained unchanged following YidC depletion.

The inner membrane associated subunits of two DMSO reductases, DmsA, DmsB, YnfE and YnfG all decreased slightly upon YidC depletion. The membrane anchor subunits were not identified.

The aerobically expressed formate dehydrogenase subunit FdoG has been shown to require the Tat translocase for translocation across the membrane (196) and upon YidC depletion, the levels of this protein did not change. All subunits of the fumarate reductase were quantified under anaerobic conditions with none of the proteins changing significantly upon YidC depletion. Under aerobic conditions FrdA was found to decrease. The catalytic subunits of the structurally-related succinate dehydrogenase remained essentially unchanged under all conditions tested. Subunits of the anaerobic glycerol-3-phosphate dehydrogenase were found to either remain unchanged or in the case of GlpB increased upon YidC depletion. The subunits of hydrogenase-2 and NarG of the nitrate reductase 1 were also not negatively affected by YidC-depletion.

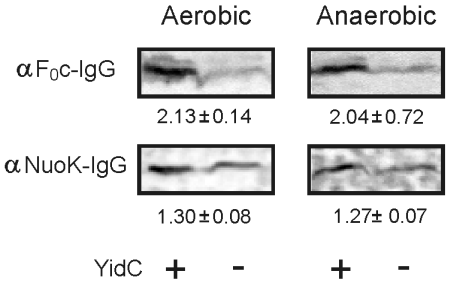

FIGURE 3. **Levels of F_oc and NuoK in the membrane decrease upon YidC depletion**. Membrane fractions were analyzed by immunoblotting using antisera directed against F_oc (upper panel) and NuoK (lower panel). The ratio of the $YidC^+/YidC^-$ signals was calculated. Indicated are the means of three separate measurements ± the standard error of the mean.

Both NADH dehydrogenases were quantified in this study with Ndh displaying a slight, increase following YidC depletion under aerobic and anaerobic conditions. The NADH-quinone oxidoreductase (also known as complex I) consists of 13 subunits in *E. coli*, 7 of which were quantified in this study. No significant changes were observed for any of the subunits of the peripheral arm of complex I upon YidC depletion. The integral membrane subunits NuoA and NuoL decreased the most upon YidC depletion with NuoA decreasing significantly in the aerobic samples. Levels of the smallest subunit of the NADH-quinone oxidoreductase, NuoK, has been previously shown to decrease upon YidC depletion (376). This was checked by immunoblot analysis and a small but consistent decrease upon YidC depletion was seen under both aerobic and anaerobic growth conditions (Figure 3, lower panel).

Stress response proteins - A number of proteins implicated in the cellular response to various stresses were quantified (Table 3). A number of these appear not to be induced under YidC depletion conditions. The Cpx sensor protein, CpxA and the Cpx-regulated protease HtpX which have been suggested to function in membrane protein quality control (440), the protease Lon, the carbon starvation protein A, lipoprotein E and YjiY which have all been implicated in the cell's response to conditions such as osmotic stress and carbon starvation as well as the so-called universal stress response proteins F and G were not induced upon YidC depletion. YjiY and Kup, which is a potassium transporter system that is upregulated under stress conditions, decreased significantly upon YidC depletion as did the sigma E protease, RseP, under anaerobic growth conditions. Sigma factor E which is involved in the cell's response to heat shock and oxidative stress response showed no induction upon YidC depletion with levels of its regulator RseB also unaffected.

In contrast, under aerobic growth conditions, YidC depletion caused a massive increase in the levels of chaperones involved in the cell response to heat shock and protein misfolding and aggregation. The chaperones ClpB, DjlA, DnaJ, DnaK, GroEL and HtpG (also referred to as Hsp90) all show an increase of between 1.7 and 4.2-fold. Levels of the cold shock protein DeaD increased by nearly 5-fold. Even more dramatic is the increase in the small heat shock proteins IbpA and B which increased approximately 16-fold. The well-characterized PspA response is also observed upon YidC depletion under aerobic conditions with Psp proteins A, B and C increasing greatly. This is however not observed when YidC depletion is performed under anaerobic growth conditions. As previously characterized, there is no PspA response and many of the other chaperones were not identified in the

TABLE 3. Proteins involved in stress responses

Gene name[a]	Protein name[b]	TMS / localization[c]	Log_2 ($YidC^+$-$YidC^-$)[d]	
			Aerobic	Anaerobic
cstA	Carbon starvation protein A	18	0.11	-0.15
cpxA	Sensor protein CpxA	2	not identified	-0.13
htpX	Probable protease HtpX	4	-0.29	-0.20
lon	ATP-dependent protease La	c	not identified	-0.22
osmE	Osmotically-induced lipoprotein E	im lp	not identified	**-0.58**
rpoE	RNA polymerase sigma-E factor	c	not quantified[e]	0.52
rseB	Sigma-E factor regulatory protein RseB	p	-0.44	not quantified
rseP	Regulator of sigma E protease	3	-0.29	**-0.89**
uspF	Universal stress protein F	c	not identified	-0.28
uspG	Universal stress protein G	c	not identified	0.18
yjiy	Inner membrane protein YjiY	17	**-0.70**	0.00
kup	Low affinity potassium transporter system protein Kup	12	**-1.00**	not identified
clpB	Chaperone protein ClpB	c	**1.93**	not quantified
djlA	DnaJ-like protein DjlA	1	**0.74**	-0.03
dnaJ	Chaperone protein DnaJ	c	**2.11**	not identified
dnaK	Chaperone protein DnaK	c, im ass	**1.59**	**0.58**
groL	60 kDa chaperonin (GroEL protein)	c	**1.11**	**0.75**
htpG	Chaperone protein HtpG	c, im ass	**1.25**	not identified
ibpA	Small heat shock protein IbpA	c	**3.97**	not identified
ibpB	Small heat shock protein IbpB	c	**4.14**	not identified
pspA	Phage shock protein A	c, im ass	**1.77**	-0.54
pspB	Phage shock protein B	1	**1.97**	-0.48
pspC	Phage shock protein C	1 ?	**2.20**	-0.32

[a] Gene designations in the UniProtKB/Swiss-Prot database for *E. coli.*
[b] Protein designations in the UniProtKB/Swiss-Prot database for *E. coli.*
[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; p, periplasm; im ass, inner membrane associated; im lp, inner membrane lipoprotein.
[d] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains.
[e] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.
Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

anaerobic samples (376). An increase was observed for the chaperones DnaK and GroEL but was much reduced compared to that observed for the aerobic samples. An increase in Dps which is involved in DNA protection during starvation was observed under anaerobic growth conditions but this protein was not identified in the aerobic data set. Levels of the chaperone DjlA remained unchanged.

Many of the chaperones identified are in fact cytoplasmic proteins and it was therefore of interest whether the increase seen in the membrane preparations represented increased levels in the cytoplasm or a sequestering of the chaperones to the membrane. Immunoblot analysis was therefore performed on cytoplasmic and membrane preparations (Figure 4). Under aerobic growth conditions, levels of IbpA were found to increase massively in the inner membrane fraction with a smaller but reproducible increase in the cytoplasmic fraction prepared from aerobically grown cells indicating that there is a sequestering of the small chaperone to the membrane. IbpA and IbpB were not identified in the anaerobic samples but immunoblot analysis of cytoplasmic and membrane fractions showed a small increase in the levels IbpA upon YidC depletion. IbpA is however, barely detectable in the cytoplasmic fraction. Under aerobic conditions the chaperone GroEL shows a similar but not as dramatic sequestering to the membrane. The increase in GroEL levels in the membrane fraction is higher upon YidC depletion than that observed in the cytoplasmic but the difference is less than that observed for IbpA. The sequestering of chaperones to the membrane was not observed for DnaK with levels increasing equally in the cytoplasmic and membrane fractions under both aerobic and anaerobic conditions. The increase was more substantial in the aerobically grown cells consistent with the data from the metabolic labeling experiments. PspA is an inner membrane-associated protein and is barely detectable in the cytoplasmic fraction isolated form aerobically grown cells (Figure 4). It is not detectable in the cytoplasmic fraction from anaerobically grown cells. The immunoblot analysis of the membrane fractions is consistent with the data from the metabolic labeling experiments.

Transporter proteins - Subunits belonging to 24 different ABC transporters were identified and quantified (Table 4). Of the 24 transporters, 19 were observed to follow that same trend upon YidC depletion under aerobic as well as anaerobic conditions. Significant decreases upon YidC deletion were observed for subunits of arginine (ArtI), dipeptide (DppA), histidine (HisJ and HisP), branched amino acid (LivF, LivG and LivP), lipoprotein-releasing (LolD and LolE), lipopolysaccharide (LptB and LptF), putrescine (PotF) and uncharacterized (YhiH) transporters.

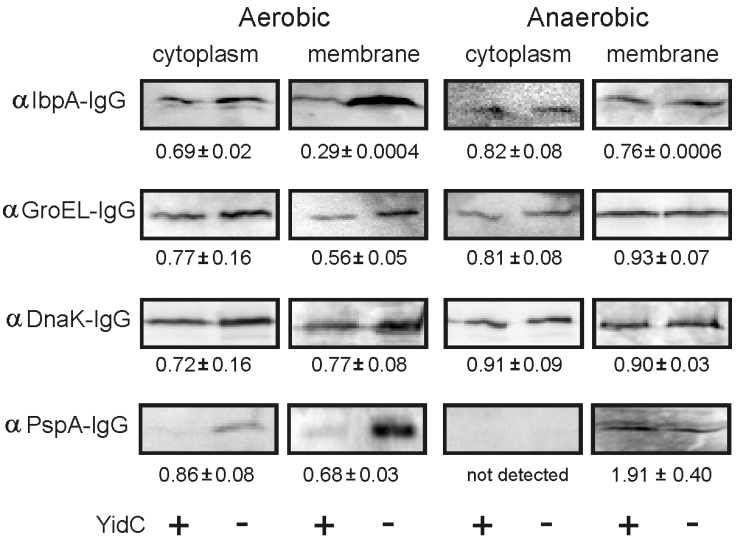

FIGURE 4. Chaperones IbpA and GroEL are sequestered to the membrane upon YidC depletion. Cytoplasmic and membrane fractions were analyzed by immunoblotting using antisera directed against IbpA, GroEL, DnaK and PspA. The ratio of the $YidC^{+}/YidC^{-}$ signals was calculated. Indicated are the means of three separate measurements ± the standard error of the mean.

Significant increases were also observed in 4 transporters i.e. the heme (CydC), methionine (MetN and MetQ) and phosphate (PstB) import systems and the multidrug resistant-like ATP-binding protein (MdlB).

For the other transporter families, fewer proteins were identified making the identification of trends upon YidC depletion difficult. None of the subunits of the AAE transporter, the amino acid/polyamine transporter 2, DcuA/DcuB transporter, inorganic phosphate (PiT), multidrug efflux, nicotinamide ribonucleotide (NR) uptake permease, sodium/potassium/calcium exchanger and xanthine/uracil permease families were observed to change upon YidC depletion. Transporter proteins AroP and CycA belonging to the APC superfamily of transporters were observed to decrease significantly following YidC depletion while PheP increased. Also, CopA of the cation transport ATPase (P-type) family, CorA which belongs to the CorA metal ion transport (MIT) family, the iron transporter FeoB, the MFS transporters KgtP and ProP and the serine/threonine transporter SstT decreased upon YidC depletion. Also levels of YjcE and YbaL belonging to the monovalent cation:protein antiporter transporter family decreased and increased respectively upon YidC depletion.

TABLE 4. Transporter proteins

Gene _name[a]	Protein name[b]	TMS / localization[c]	Log_2 ($YidC^+$-$YidC^-$)[d] Aerobic	Anaerobic
AAE transporter family				
ybjL	Putative transport protein ygjL	10	-0.50	not identified
ABC transporters				
artI	Arginine-binding periplasmic protein 1	p	not quantified[e]	**-2.86**
artJ	Arginine-binding periplasmic protein 2	p	not identified	-0.03
artP	Arginine transporter ATP-binding protein ArtP	c, im ass	-0.24	-0.29
cydC	ATP-binding/permease protein CydC	6	0.21	**0.59**
cydD	ATP-binding/permease protein CydD	6	0.39	0.15
cysA	Sulfate/thiosulfate import ATP-binding protein CysA	c, im ass	-0.57	-0.25
dppA	Periplasmic dipeptide transport protein	p	**-1.61**	-0.20
dppB	Dipeptide transport system permease DppB	7	**-0.61**	0.43
dppD	Dipeptide transport ATP-binding protein DppD	c, im ass	-0.51	-0.22
dppF	Dipeptide transport ATP-binding protein DppF	c, im ass	**-0.61**	-0.24
glnQ	Glutamine transport ATP-binding protein	c, im ass	0.01	0.02
hisJ	Histidine-binding periplasmic protein	p	-0.43	0.06
hisP	Histidine transport ATP-binding protein HisP	c, im ass	**-0.71**	-0.16
livF	High-affinty branched amino acid transport ATP-binding protein LivF	c, im ass	-0.35	**-0.64**
livG	High-affinty branched amino acid transport ATP-binding protein LivG	c, im ass	-0.28	**-0.63**
livJ	Leu/Ile/Val-binding protein	p	**-1.13**	0.03
livK	Leucine-specific-binding protein	p	-0.14	-0.08
lolC	Lipoprotein-releasing system transmembrane protein LolC	4	-0.39	not identified
lolD	Lipoprotein-releasing system ATP-binding protein LolD	c, im ass	-0.54	not identified
lolE	Lipoprotein-releasing system transmembrane protein LolE	4	**-0.80**	-0.25

lptB	Lipopolysaccharide export system ATP-binding protein LptB	c, im ass	**-0.60**	-0.39
lptF	Lipopolysaccharide export system permease protein LptF	6	-0.52	-0.47
malK	Maltose/maltodextrin import ATP-binding protein MalK	c, im ass	not quantified	-0.53
mdlB	Multidrug resistant-like ATP-binding protein MdlB	6	-0.22	not identified
metI	D-methionine transport system permease protein MetI	5	-0.02	0.00
metN	Methionine import ATP-binding protein MetN	c, im ass	**0.80**	-0.18
metQ	D-methionine -binding lipoprotein MetQ	lp	**0.60**	0.29
mglA	Galactose/methyl galactoside import ATP-binding protein MglA	c, im ass	not identified	-0.15
mglB	D-galactose-binding periplasmic protein	p	not identified	-0.51
msbA	Lipid A export ATP-binding/permease protein MsbA	5	-0.19	0.07
potA	Spermidine/putrecine import ATP-binding protein PotA	c. im ass	-0.02	-0.43
potF	Putrescine-binding periplasmic protein	p	**-0.93**	not identified
potG	Putrescine transport ATP-binding protein	c, im ass	-0.19	not identified
pstB	Phosphate import ATP-binding protein PstB		**0.90**	0.23
rbsA	Ribose import ATP-binding protein RbsA	c, im ass	0.01	-0.13
rbsB	D-ribose-binding periplasmic protein	p	not quantified	0.16
rbsC	Ribose transport system permease protein RbsC	6	not quantified	-0.20
yadG	Uncharacterized ABC transporter ATP-binding protein YadG	c, im ass	0.14	0.22
yecC	Uncharacterized amino-acid ABC transporter ATP-binding protein YecC	c, im ass	-0.38	-0.30
yecS	Inner membrane amino-acid ABC transporter permease protein YecS	3	not quantified	-0.49
yhiH	Uncharacterized ABC transporter ATP-binding protein	6	**-0.72**	not identified
yjjK	Uncharacterized ABC transporter ATP-binding protein YjjK	c, im ass	not identified	-0.39
ynjD	Uncharacterized ABC transporter ATP-binding protein YnjD	c, im ass	not identified	-0.14
yojI	ABC transporter ATP-binding protein YojI	c, im ass	-0.36	not identified
yrbC	Protein YrbC	p	not identified	-0.11
yrbD	Uncharacterized protein YrbD	p	0.36	-0.03
yrbF	Uncharacterized ABC transporter ATP-binding protein YrbF	c, im ass	0.44	-0.33

TABLE 4. continued

Amino acid/polyamine transporter 2 family				
sdaC	Serine transporter	11	-0.50	not identified
APC superfamily transporters				
aroP	Aromatic amino acid transporter protein AroP	12	-0.47	-0.26
cycA	D-alanine/D-serine/glycine transporter	12	**-0.98**	not identified
lysP	Lysine-specific permease	12	-0.44	not identified
pheP	Phenylalanine-specific permease	12	**1.39**	not identified
ydgI	Putative arginine/ornithine antiporter	12	not quantified	0.11
Cation transport ATPase (P-type) family				
copA	Copper-exporting P-type ATPase A	8	**-0.74**	not identified
mgtA	Magnesium-transporting ATPase, P-type 1	10	0.42	0.04
zntA	Lead, cadmium, zinc and mercury-transporting ATPase	7	-0.40	not identified
CorA metal ion transporter (MIT) family				
corA	Magnesium transport protein CorA	2	**-1.09**	**-0.58**
zntB	Zinc transport protein ZntB	2	0.06	not identified
DcuA/dcuB transporter family				
dcuA	Anaerobic C4-dicarboxylate transporter DcuA	10	**0.70**	0.13
dcuB	Anaerobic C4-dicarboxylate transporter DcuB	10	not quantified	0.14
FeoB family				
feoB	Ferrous iron transporter protein B	13	**-1.59**	not identified
FNT transporter family				
focA	Probable formate transporter 1	6	not quantified	-0.19
Inorganic phosphate transporter (PiT) family				
pitA	Low-affinity inorganic phosphate transporter 1	10	-0.17	-0.31
LptC family				
lptC	Lipolysaccharide export system protein LptC	1	-0.03	0.06
MFS transporters				
galP	Galactose-proton symporter	12	not quantified	0.05
glpT	Glycerol-3-phosphate transporter	12	not identified	-0.24

kgtP	Alpha-ketoglutarate permease	12	**-0.72**	not quantified
proP	Proline/betaine transporter	12	-0.45	-0.23
yhjE	Inner membrane metabolite transport protein YhjE	12	not quantified	0.37
Monovalent cation:protein antiporter transporter families 1 and 2				
yjcE	Uncharacterized Na^+H^+ exchanger YjcE	12	-0.57	-0.09
ybaL	Inner membrane protein ybaL	13	**1.22**	not identified
Multidrug resistance				
acrA	Acriflavine resistance protein A	im lp	0.44	0.13
acrB	Acriflavine resistance protein B	12	-0.37	-0.17
emrA	Multidrug resistance protein A	1	-0.40	not quantified
macA	Macrolide-specific efflux protein MacA	p, im ass	-0.12	not identified
mdtA	Multidrug resistance protein MdtA	p, im ass	-0.27	not identified
ybhG	UPF0194 membrane protein YghG	p, im ass	0.19	0.38
Nicotinamide ribonucleoside (NR) uptake permease family				
pnuC	Nicotinamide riboside transporter PnuC	6	not identified	-0.18
Sodium:dicarboxylate (SDF) symporter family				
sstT	Serine/threonine transporter SstT	8	**-1.78**	-0.44
ydjN	Uncharacterized symporter YdjN	10	-0.35	0.11
Sodium/potassium/calcium exchanger family				
chaA	calcium/proton antiporter	11	-0.12	not identified
Xanthine/uracil permease family				
yjcD	Putative permease YjcD	13	-0.38	-0.15

[a] Gene designations in the UniProtKB/Swiss-Prot database for *E. coli*.

[b] Protein designations in the UniProtKB/Swiss-Prot database for *E. coli*.

[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; p, periplasm; im ass, inner membrane associated; im lp, inner membrane lipoprotein.

[d] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains.

[e] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.

Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

Membrane protein quality control - The ATP-dependent protease HflB (also known as FtsH) along with its regulator modules HflC and HflK were quantified in both the aerobic and anaerobic datasets (Table 5). HflB has been implicated in the proteolysis of misfolded or uncomplexed proteins (10,11) as well as having chaperone function during protein translocation (441). A more pronounced increase of these 3 proteins was observed in the aerobic compared to the anaerobic conditions and a significant increase upon YidC depletion was only observed for HflK.

Cell division and cell shape - A number of proteins involved in the cell division and determination of cell shape were identified in this study with the lipoprotein NlpI and the cell division protein FtsX observed to decrease upon YidC depletion but only in the aerobic samples (Table 5). The rod shape-determining protein MreB increased significantly following YidC depletion under aerobic growth conditions. Under anaerobic conditions, no changes were observed.

Cofactor biosynthesis - The synthesis of heme cofactors involved a myriad of proteins, 3 of which were quantified in this study. Levels of the protoporphyrin oxidase HemG decreased significantly upon YidC depletion but was only quantified under anaerobic conditions (Table 5).

Penicillin binding proteins - The penicillin binding proteins DacA and MrdA showed significant changes upon YidC depletion with protein levels increasing under aerobic conditions.

Miscellaneous - A further 43 integral membrane proteins were identified which did not fit into any of the above categories mentioned above (Table 5 and Supplementary Table 1). In Table 5, proteins which exhibited a significant change upon YidC depletion are shown. The biopolymer transport protein, ExbB, decreased by a factor of 4 upon YidC depletion under aerobic conditions, the largest decrease observed after YidC. The membrane-spanning proteins TolQ and TolR too showed a decrease upon YidC depletion as did GtrB. The uncharacterized proteins YbbM, YebE, YegH, YjiG, YpfJ and YqjE all showed significant decreases in protein levels following YidC depletion while only protein YcjF showed an increase under aerobic and anaerobic growth conditions.

Proteases - Six membrane-embedded/-associated proteases were further quantified none of which showed significant changes following YidC depletion (Supplementary Table 1).

Lipoproteins - In total, 10 inner membrane-associated lipoproteins were identified (Supplementary Table 1). No general trend of an increase or decrease in

levels was observed. RlpA and YdcL were observed to decrease significantly upon YidC depletion but only in the aerobic samples.

Sensor proteins - Four membrane-embedded sensor proteins were identified (Supplementary Table 1) with only PhoQ changing significantly with an increase in protein levels upon YidC depletion.

Ion channels - In total, 5 ion channels were identified. MscS decreased significantly following by YidC depletion with the MscS-related protein YjeP decreasing only 1.4-fold (Supplementary Table 1). The other ion channels were unaffected by YidC depletion. The mechanosensitive channel of large conductance MscL, which has been shown to depend on YidC for insertion and/or oligomerization (145,371) was not identified.

Metabolism - For the membrane-embedded/–associated protein involved in metabolism, no significant changes were observed upon YidC depletion under either aerobic or anaerobic growth conditions (Supplementary Table 1).

PTS system - Also for the membrane components of the PTS systems identified, no changes following YidC depletion were observed (Supplementary Table 1).

Outer membrane proteins - A total of 47 outer membrane proteins, 32 in the aerobic and 41 in the anaerobic dataset, were identified in the inner membrane preparation (Supplementary Table 2). The levels of the majority of the proteins did not change upon YidC depletion. Levels of the transglycosylase EmtA, the receptors FhuE and Fiu, the lipoprotein NlpD, the outer membrane protein OmpC and the predicted proteins YcfM and YgiB did however decrease significantly while the levels of the outer membrane protein Slp increased upon YidC depletion. In the case of EmtA, YcfM and YgiM the decrease was only observed in the aerobic sample.

Periplasmic proteins - Also identified in the inner membrane preparation were a few periplasmic proteins. In total 18 were identified with 11 in the aerobic and 16 in the anaerobic dataset (Supplementary Table 3). Of the 18 periplasmic proteins quantified, 8 were observed to decrease significantly upon YidC depletion. The L-asparaginase AnsB, the carboxypeptidase DacB, the proteases DegP and DegQ, the cysteine-binding protein FliY, the galactose-binding protein OppA as well as the chaperones Skp and SurA all decreased significantly under YidC-depleting conditions. An increase in protein levels was not observed.

Proteins with an unknown cellular location - In addition to the proteins mentioned above, a further 6 proteins were identified to which no cellular

TABLE 5. Other proteins identified in this study

Gene name[a]	Protein name[b]	TMS / localization[c]	Log2 (YidC$^+$-YidC$^-$)[d] Aerobic	Anaerobic
Membrane protein quality control				
hflB	Cell division protein FtsH	2	0.53	0.22
hflC	Protein HflC	1	0.52	0.13
hflK	Protein HflK	1	**0.60**	0.14
Cell division and cell shape				
ftsE	Cell division ATP-binding protein FtsE	c, im ass	not quantified[e]	0.46
ftsN	Cell division protein FtsN	1	0.03	0.01
ftsX	Cell division protein FtsX	4	**0.59**	-0.14
ftsZ	Cell division protein FtsZ	c, im ass	-0.01	0.18
nlpI	Lipoprotein NlpI	lp	**-0.78**	-0.06
zipA	Cell division protein ZipA	1	not quantified	0.10
ftsI	Peptidoglycan synthetase FtsI	1	-0.05	not identified
minD	Septum site-determining protein MinD	c, im ass	0.02	0.24
damX	Protein Damx	1	0.07	0.08
mreB	Rod shape-determining protein MreB	c	**0.74**	0.35
mreC	Rod shape-determining protein MreC	amb	-0.12	not identified
mrcB	Penicillin-binding protein	1	-0.05	0.15
Cofactor biosynthesis				
hemY	Protein HemY	2	0.16	0.25
hemX	Putative uroporphyrinogen-III C-methyltransferase	1	0.17	0.30
hemG	Protoporphyrin oxidase	amb	not quantified	**-0.74**
Penicillin binding proteins				
dacA	D-alanyl-D-alanine carboxypeptidase DacA	1	**0.59**	0.02
mrdA	Penicillin-binding protein 2	p, im ass	0.50	not identified

Miscellaneous				
exbB	Biopolymer transport protein ExbB	3	**-2.06**	not identified
lnt	Apolipoprotein N-acyltransferase	6	**-0.61**	0.30
tolQ	Protein TolQ	3	**-0.67**	**-0.76**
tolR	Protein TolR	1	not identified	**-0.94**
ybbM	UPF0014 inner membrane protein YbbM	7	**-1.14**	not identified
ycjF	UPF0283 membrane protein YcjF	3	**1.00**	0.55
yebE	Inner membrane protien YebE	1	not identified	**-0.94**
yegH	UPF0053 protein YegH	5	**-0.59**	-0.36
yfdH	Bactoprenol glucosyl transferase homolog from prophage CPS-53	2	**-0.68**	-0.52
yhdP	Uncharacterized protein YhdP	1	**0.58**	-0.25
yjiG	Inner membrane protein YjiG	4	not identified	**-1.88**
ypfJ	Uncharacterized protein YpfJ	1	**-0.81**	not identified
yqjE	Inner membrane protein YqjE	2	**-0.69**	not quantified

[a] Gene designations in the UniProtKB/Swiss-Prot database for *E. coli.*

[b] Protein designations in the UniProtKB/Swiss-Prot database for *E. coli.*

[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; p, periplasm; im ass, inner membrane associated; im lp, inner membrane lipoprotein; amb, ambiguous.

[d] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains.

[e] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.

Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

localization could be assigned (Supplementary Table 4). Levels of the kinase AnmK and the uncharacterized protein YncE decreased significantly while the uncharacterized protein YhiK increased upon YidC depletion.

Ribosomal and cytoplasmic proteins - Only a further 8 cytoplasmic proteins exhibited significant changes upon YidC depletion and these are shown in Supplementary Table 5. Levels of aldehyde-alcohol dehydrogenase, AdhE, and the malate:quinone oxidoreductase protein Mqo decreased upon YidC depletion while levels of the citrate lyase alpha and beta chains, Ef-G, MetF and the uncharacterized proteins YjiL and YqjD increased. In total, 28 ribosomal proteins were quantified. Under aerobic growth conditions, the levels of the 50S ribosomal subunit proteins L11 and L20 and the 30S ribosomal subunit proteins S6 and S7 in the membrane fraction increased following YidC depletion (Supplementary Table 6). Although not to a significant level, under aerobic conditions all of the ribosomal proteins quantified in this study increased while this trend was not observed under anaerobic conditions following YidC depletion.

Isolation of protein aggregates - The depletion of YidC resulted in a massive upregulation of cytoplasmic chaperones often associated with protein aggregation following heat shock, such as IbpA, IbpA and HtpG. This response was not pronounced when YidC was depleted under anaerobic growth conditions. It was therefore of interest to determine whether the different growth conditions affects the amount of protein aggregation or whether the cell responds differently depending on the availability of oxygen.

Using selective solubilization in the detergent NP-40, aggregates were isolated from $YidC^{+}$ and $YidC^{-}$ strains grown under the same conditions as those used for the proteomic analysis. The protein aggregates were quantified and expressed as a percentage of the total cellular protein (Figure 5). Under aerobic conditions the amount of protein aggregates increased 1.6-fold while under anaerobic conditions by 1.25-fold. In total, 28 proteins were identified, none of which were integral membrane proteins (Table 6). Of the 28, 6 were outer membrane proteins, 2 periplasmic proteins and the rest were cytoplasmic proteins with the exception of the chaperone DnaK which is associated with the inner membrane. Several proteins involved in the stress responses were identified. The chaperone IbpA was identified as well as Dps, which plays a role in DNA protection, and the periplasmic protein Spy, which responds to envelope stress. DnaK and IbpA increased in response to YidC depletion under aerobic conditions (Table 3) while Dps was observed to increase under anaerobic conditions. Spy was not identified

in the metabolic labeling study.

Mislocalized proteins included the outer membrane proteins A, C, X and Slp. Also protease 7 (OmpT), the predicted outer membrane lipoprotein YfiO and the periplasmic proteins DegP and SurA were identified in the aggregates. In both the aerobic and anaerobic datasets, levels of outer membrane protein C in the membrane preparation decrease significantly (Supplementary Table 2) while for OmpA, OmpT and OmpX no negative effect was observed. Levels of DegP and SurA also decreased upon YidC depletion (Supplementary Table 3). In contrast, Slp which is induced by carbon starvation or slow growth, was observed to increase upon YidC depletion (Supplementary Table 2). A large number of ribosomes were identified in the aggregates as well as prolyl-tRNA synthetase and EF-Tu. Also the cytoplasmic proteins AceE, GlnA and GlgA which are involved in metabolism, the lactose repressor LacI and the uncharacterized proteins YggR and YjdP were identified in the protein aggregates. MreB, which plays a role in the determination of cell shape in *E. coli* and which was observed to increase upon YidC depletion (Table 5), was also identified in the aggregates.

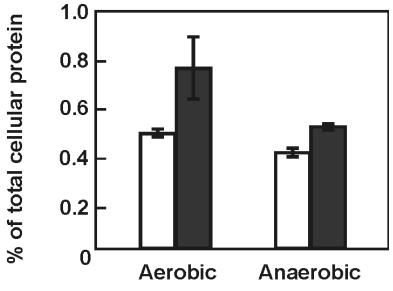

FIGURE 5. YidC depletion causes the accumulation of protein aggregates in the cell. The amount of aggregates isolated is expressed as a percentage of the total cellular protein. Protein aggregates isolated from $YidC^+$ (white bars) and $YidC^-$ (black bars) strains under aerobic and anaerobic growth conditions are compared. Data points are the mean of three separate measurements. The bars indicate the standard error of the mean.

Coimmunoprecipitation with His-YidC Oxa1 has been isolated as a complex with *in vitro*-synthesized Atp9 (homologous to F_oc) as well as with the entire F_1F_o ATP synthase suggesting a role for Oxa1 in the assembly of the protein complex (225). In *E. coli, in vitro*-synthesized F_oc has been shown to co-purify with YidC suggesting that it too contacts its substrates and remains briefly associated with them (258), while in *Bacillus subtilis*, the entire F_1F_o ATP synthase can be found to be associated with the YidC homologs SpoIIIJ and YqjG (402). Overexpression of YidC from plasmid pEH1YidC or pTrcYidC was induced with 0.5 mM IPTG and IMVs containing His-tagged YidC or wild type YidC were purified on a Ni^{2+}-NTA column. Protein profiles were compared and protein bands specific for purification with His-YidC were excised, digested with trypsin in gel followed by LC-MS/MS.

TABLE 6. Proteins contained in aggregates

Gene name[a]	Protein name[b]	localization[c]
Stress response		
dnaK	Chaperone protein DnaK	c, im ass
ibpA	Small heat shock protein IbpA	c
dpsE	DNA protection during starvation protein	c
spy	Spheroplast protein Y	p
Cell division and cell shape		
mreB	Rod shape-determining protein MreB	c
Metabolism		
aceE	Pyruvate dehydrogenase E1 component	c
glnD	[Protein-PII] uridylyltransferase	c
glgA	Glycogen synthase	c
Regulation		
lacI	Lactose operon repressor	c
Protein synthesis		
proL	Prolyl-tRNA synthetase	c
rplD	50S ribosomal protein L4.	c
rplE	50S ribosomal protein L5.	c
rplF	50S ribosomal protein L6.	c
rplI	50S ribosomal protein L9.	c
rplN	50S ribosomal protein L14.	c
rplW	50S ribosomal protein L23.	c
rpsI	30S ribosomal protein S9.	c
tufA	Elongation factor Tu	c
Periplasmic proteins		
degP	Protease do	p
surA	Chaperone surA	p
Outer membrane proteins		
ompA	Outer membrane protein A	om
ompC	Outer membrane protein C	om
ompT	Protease 7	om
ompX	Outer membrane protein X	om
slp	Outer membrane protein Slp	om
yfiO	UPF0169 lipoprotein YfiO	om
Unknown function		
yggR	Uncharacterized protein YggR	c
yjdP	Uncharacterized protein YjdP	amb

[a] Gene designations in the UniProtKB/Swiss-Prot database for *E. coli.*
[b] Protein designations in the UniProtKB/Swiss-Prot database for *E. coli.*
[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; im ass, inner membrane associated p, periplasm; om, outer membrane.

30 proteins were identified in anaerobic sample while only 7 were identified in the aerobic sample (Table 7). Of the 30 proteins found to co-purify with His-YidC in the anaerobic sample, 20 were inner membrane or membrane-associated proteins and 10 were cytoplasmic proteins. Several proteins involved in energy transduction were identified namely CyoB, a hydrogenase-2 operon protein, HybA, Ndh and NuoC/D. Also, the chaperone DnaJ was identified. Transporter proteins CorA, MetN, PutP and the uncharacterized transporter YeeF were too found to copurify with His-YidC. The quality control proteins HflB, HflC and HflK were found associated with His-YidC as previously shown (493). The HflC-related protein, QmcA was also identified in the anaerobic sample. The inner membrane sensor protein RstB, protein HemY which is involved in heme biosynthesis, the uncharacterized ion channel YbdG, WaaA which is involved in biosynthesis and the predicted membrane proteins YccS and YcjF were all found to copurify with His-YidC. Penicillin-binding protein 2, MrdA, which is associated with the inner membrane on the periplasmic side was too found copurified with His-YidC. Also co-purified with His-YidC was the integral membrane protein MraY as well as the rod shape-determining protein MreB, which are both involved in the determination of cell shape. Other cytoplasmic proteins copurified with YidC were AceF, IlvA, SucB and ZdhA which all play a role in metabolism, SlyD which is involved in protein folding, the transcriptional regulator Crp, ribonuclease E, EF-Tu and the 30S ribosomal protein S2.

When His-YidC overexpression was performed under aerobic growth conditions, only 7 proteins were identified, 4 of which were inner membrane proteins and 3 cytoplasmic proteins. The energy transducing proteins CyoA and CyoB were identified as well as Ndh. Also the sensor protein RstB was found to copurify with His-YidC. In addition the cytoplasmic proteins SlyD, Crp and ribonuclease E were co-isolated with His-YidC when overexpressed under aerobic growth conditions.

DISCUSSION

E. coli contains a highly branched respiratory chain which allows this bacteria to grow both in the presence of oxygen as well as in the presence of other inorganic electron acceptors when oxygen is unavailable. There is a multitude of primary dehydrogenases, terminal reductases and quinones and so, depending on the growth conditions, a large variability in the composition of respiratory chains is observed (488). This makes *E. coli* a good candidate for the study of respiratory chain biogenesis, and in particular the role that YidC plays in this process. In this

TABLE 7. Identification of co-purifying proteins

Gene name[a]	Protein name[b]	Localization[c]	Aerobic	Anaerobic
Membrane biogenesis				
yidC (oxaA)	Inner membrane protein oxaA	im	+	+
Energy transduction				
cyoA	Ubiquinol oxidase subunit 2	im	+	-
cyoB	Ubiquinol oxidase subunit 1	im	+	+
hybA	Hydrogenase-2 operon protein	im	-	+
ndh	NADH dehydrogenase	im	+	+
nuoCD	NADH-quinone oxidoreductase subunit C/D	c, im ass	-	+
Stress response				
dnaJ	Chaperone protein dnaJ	c, im ass	-	+
Transport				
corA	Magnesium transport protein corA	im	-	+
metN	Methionine import ATP-binding protein MetN	c, im ass	-	+
putP	Sodium/proline symporter	im	-	+
yeeF	Inner membrane transport protein YeeF	im	-	+
Quality control				
hflB	Cell division protein FtsH	im	-	+
hflC	Protein HflC	im	-	+
hflK	Protein HflK	im	-	+
qmcA	Protein QmcA	im	-	+
Sensor proteins				
rstB	Sensor protein RstB	im	+	+
Cell division and cell shape				
mraY	Phospho-N-acetylmuramoyl-pentapeptide-transferase	im	-	+
mreB	Rod shape-determining protein MreB	c	-	+
Cofactor biosynthesis				
hemY	Protein hemY	im	-	+

Ion channels				
ybdG	Uncharacterized protein YbdG	im	-	+
Metabolism				
aceF	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	c	-	+
ilvA	Threonine dehydratase biosynthetic	c	-	+
sucB	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	c	-	+
xdhA	Xanthine dehydrogenase molybdenum-binding subunit	c	-	+
Penicillin binding proteins				
mrdA	Penicillin-binding protein 2	p, im ass	-	+
Protein folding				
slyD	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	c	+	-
Transcription regulation				
Crp	Catabolite gene activator	c	+	+
Endonuclease				
rneE	Ribonuclease E	c	+	+
Protein synthesis				
tufA	Elongation factor Tu	c	-	+
rpsB	30S ribosomal protein S2	c	-	+
Lipopolysaccharide biosynthesis				
waaA	3-deoxy-D-manno-octulosonic-acid transferase	im	-	+
Unknown function				
yccS	Inner membrane protein YccS	im	-	+
ycjF	UPF0283 membrane protein YcjF	c	-	+

[a] Gene designations in the UniProtKB/Swiss-Prot database for *E. coli.*
[b] Protein designations in the UniProtKB/Swiss-Prot database for *E. coli.*
[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; im, inner membrane; im ass, inner membrane associated p, periplasm.

study we compared the effect of YidC depletion under aerobic and anaerobic conditions in order to broaden the range of energy transducing complexes identified since members of the Oxa1/Alb3/YidC family have been implicated in the biogenesis of numerous protein and protein complexes of the respiratory chain. Subunits of 12 characterized respiratory complexes as well as two putative respiratory chain components were quantified. Although YidC has a well-characterized role in the insertion of F_oa and F_oc of the F_1F_o ATP synthase (499,499,543,544), the effect of YidC depletion on the other subunits of the complex was not significant. Although small decreases were observed for all the subunits quantified, the decrease was much smaller than the 2-fold decrease observed in the immunoblot analysis of F_oc . It appears that assembly of the subunits at the membrane can occur even when levels of the c-ring are reduced. YidC may also have, in addition to its insertase function, a role in the assembly of the F_1F_o ATP synthase. In yeast, Oxa1 is not essential for the insertion of Atp9 (F_oc) but acts post-translationally as a chaperone mediating the interaction of the Atp9 oligomeric ring with Atp6 (F_oa). In *Bacillus subtilis,* two YidC homologs, SpoIIIJ and YqjG, are present. Similarly to YidC, either protein alone is sufficient to catalyze the *in vitro* insertion of *B. subtilis* F_oc (402). In addition, SpoIIIJ and YqjG form a complex with the entire F_1F_o ATP synthase, suggesting that these proteins have a role in the final assembly of this protein complex (402). Members of the Oxa1/Alb3/YidC family have been implicated in the biogenesis of complex I (331,454) and in this study 7 of the 13 subunits of complex I (NADH-ubiquinone oxidoreductase) in *E. coli* were quantified, with the membrane embedded subunits exhibiting the greatest negative effect. Complex I biogenesis has been proposed to include the formation of intermediate complexes resembling the 3 functional domains which in turn assemble to form the holoenzyme. The NADH dehydrogenase subcomplex (NuoEFG) can form independently of the other subunits (428) and indeed changes in the levels of subunits F and G observed upon YidC depletion are very subtle. This suggests that the NADH dehydrogenase fragment assembles and is targeted to the membrane even when biogenesis of the membrane part of complex I is impaired. In a previous study, we showed that levels of the smallest membrane subunit, NuoK, decrease upon YidC depletion under anaerobic conditions (376). It has yet to be elucidated the exact role YidC plays in the biogenesis of this and possibly other membrane subunits of complex I.

For most of the subunits of energy transducing complexes identified in this study, no major changes were observed. This included the well-characterized YidC-

dependent protein CyoA, although the levels of this membrane protein were reduced slightly. CyoB does not require CyoA for insertion and stability in the membrane (328) and it is unclear why although unaffected under aerobic conditions, levels of this protein decrease in the anaerobic samples. The only significant negative effect upon YidC depletion was observed for FrdA under aerobic conditions. However, no large decrease is seen for any of the fumarate reductase subunits identified in the anaerobic samples. In a previous study, using antibodies we showed that levels of the catalytic subunits of the fumarate reductase, FrdA and FrdB, decrease upon YidC depletion as does the fumarate reductase activity of cells grown in fumarate-containing medium (376). Apparently, this reduction is too small to detect with the proteomic method applied.

Both formate dehydrogenase-O and hydrogenase-2 have been shown to rely on the Sec-independent Tat translocation pathway and these proteins are not affected by YidC depletion. This indicates that although changes in the levels of TatB and TatE are observed upon YidC depletion, the Tat translocation pathway is still functional. The levels of TatA are unaffected by YidC depletion and since TatA and TatE have overlapping functions (415), the slight decrease in TatE levels could be compensated for by TatA.

Although some components of the Sec translocase showed a decrease in levels upon YidC depletion, this decrease was not significant and for most outer membrane proteins no negative effect was observed indicating that protein translocation was still functional. Secondary effects, such as the reduction of the proton motive force as reported earlier (499), should however always be taken into consideration when essential genes, such as YidC, are depleted in the cell. Levels of SecA associated with the membrane increased when YidC depletion was performed under aerobic growth conditions. SecA translation has been shown to be upregulated in response to defects in protein secretion (303,425). This is modulated by SecM which is located upstream of SecA(330). Taken together with the observation that for most outer membrane proteins no decrease is observed, this indicates that there is a certain degree of secretion hindrance when YidC is depleted from the cell but a complete blockage of the Sec translocase does not occur. SecA is not upregulated in anaerobically grown cells possibly indicative of a less severe secretion defect.

Although the changes in membrane protein levels described above were subtle, a dramatic increase was observed in the chaperone-mediated stress response and

interestingly, this response was limited in the aerobically grown cells. The response appears to be specifically to the misfolding and aggregation of proteins in the cytoplasm. The PspA response is a well-studied response to YidC depletion (502). The phage shock protein (psp) operon (*pspABCE*) is induced by many stress factors such as bacteriophage infection, ethanol, heat and osmotic shock and has an unknown function in the maintenance of the proton motive force. (254). It has been previously published that PspA does not play a role in the response to osmotic stress (518) or YidC depletion (376) under anaerobic conditions. However, using pIV-induction of *psp* expression, PspA induction was seen under aerobic, microaerobic and anaerobic growth conditions (232). pIV is an outer membrane secretin that when overexpressed, localizes to the inner membrane impairing the cell membrane integrity and resulting in *psp* expression. Following controversy regarding the role of the ArcAB system, which regulates the transition from aerobic to anaerobic respiration and fermentation, in the induction of PspA (233,437), it has been demonstrated that only under microaerobic conditions, do ArcA and B play a role in the pIV induction of the PspA response (232). Under anaerobic conditions, neither ArcAB nor PspBC are essential for the induction of PspA. The PspBC-dependence of PspA induction is also dependent on the kind of stress the cells are exposed to. For example, PspA induction following extreme heat shock and CCCP treatment is PspBC-independent while it is only partially dependent on PspBC following ethanol treatment and osmotic shock. The precise nature of the signals that induce *psp* expression remains to be determined but does appear to be effected by the nature of the stress as well as the availability of oxygen. In the cases where *psp* expression is not induced, it is unclear how *E. coli* deals with such stresses differently under anaerobic conditions and what other stress-related proteins replace the PspA response.

Since YidC depletion causes the aggregation of proteins in the cytoplasm the observation that levels of the small chaperones IbpA and IbpB increase under aerobic growth conditions is not surprising. This response has been shown during heat shock and oxidative stress (253) as well as to overexpression of heterologous proteins (13). The increase in IbpA levels was most pronounced in the membrane compared to the cytoplasmic fractions. This may indicate protein aggregation at the inner membrane surface. IbpA, together with IbpB, associates with aggregated proteins to stabilize and protect them from irreversible denaturation and extensive proteolysis (261). Aggregated proteins bound to the IbpAB complex are more efficiently refolded and reactivated by ATP-dependent chaperone systems such as

ClpB, HtpG and DnaJ, DnaK and GlpE (315). ClpB and HtpG have been implicated in *de novo* protein folding in mildly stressed cells (466) and may assist the cell under conditions in which membrane proteins are not efficiently inserted. Levels of the chaperones DnaJ and DnaK too increased upon YidC depletion and could be involved in the refolding of aggregated proteins to allow protein translocation to continue. GroEL has been shown to prevent misfolding and promote refolding and proper assembly of unfolded polypeptides generated under stress conditions rather than the refolding of aggregated proteins (185) and under aerobic growth conditions a greater increase in GroEL levels was observed in the membrane than in the cytoplasmic fractions. This suggests that GroEL is sequestered to the membrane upon YidC depletion possibly to deal with proteins aggregating at the inner membrane surface. Under anaerobic conditions the DnaJ-like protein, DjlA, did not change. Levels of IbpA, DnaK and GroEL were observed to increase slightly but this was not comparable to the response observed in the aerobically grown cells and no specific sequestering to the membrane was observed. Also, in contrast to the anaerobic samples, the ribosomes present in the membrane fraction in the aerobic samples accumulated upon YidC depletion. This too could be indicative of a defect in protein insertion and/or translocation via the Sec translocase or as result of ribosomes targeted to the membrane but unable to transfer the protein nascent chains to YidC.

The extent of protein aggregation in YidC-depleted cells was therefore investigated. Protein aggregation was observed upon YidC depletion under both aerobic and anaerobic growth conditions. Proteins identified in the aggregates included OmpC and the periplasmic proteins DegP and SurA, whose levels in the membrane were found to decrease upon YidC depletion. This could be indicative of a defect in secretion in YidC-depleted cells. As predicted by the cross-beta prediction program Tango (http://tango.embl.de), the signal sequences of OmpA, C, F and X are prone to aggregation. Accumulation of one precursor outer membrane protein may cause the aggregation of the other porins. Although a major difference in the number and extent of chaperones induced upon YidC depletion was observed, the difference in the extent of aggregation between the aerobically and anaerobically grown cells was small. This suggests that *E. coli* may contain yet to be characterized chaperone systems that respond to protein aggregation under anaerobic conditions, that the cell is less able to respond to protein aggregation under these conditions or that the basal levels of the chaperones identified is sufficient for responding to protein aggregation under

anaerobic growth conditions. Alternatively, there is a threshold of protein aggregation that needs to be reached in order to elicit the chaperone response observed under aerobic conditions.

A similar chaperone response to that under aerobic conditions is observed when SecE is depleted from *E. coli* (21). However in contrast to SecE depletion, YidC depletion causes an increase in the levels of the membrane protein quality control proteins HflBCK. This is evidence that protein misfolding occurs not only in the cytoplasm but also in the membrane upon YidC depletion. The increase was more pronounced under aerobic than anaerobic conditions. Proteins FtsH (or HflB), HflC and HflK were also shown to copurify with YidC in this study and others (493). This suggests that these proteins act early on in membrane biogenesis. YidC has been implicated in the correct folding of membrane proteins and their stability and incorporation into protein complexes as illustrated by studies on LacY (326) and MalF (514). It may be in this capacity that YidC interacts with HflBCK to ensure correct folding of membrane proteins.

Although the current study was performed on inner membrane protein preparations there was some contamination of other proteins. Cytoplasmic proteins are very well-suited to trypsin digestion and the large number identified does not necessarily reflect a large quantity of cytoplasmic proteins present in the sample. Also it is not clear whether the presence of outer membrane proteins in the inner membrane preparation is a contamination of outer membrane proteins because of the large quantity of porins such as OmpF and OmpC in the membrane (316) or a result of proposed contacts made between the inner and outer membrane (107).

The proteomic analysis of membrane proteins remains challenging owing to their poor digestion by trypsin and unsuitability to conventional proteomic separation methods. The metabolic labeling and subsequent separation of trypsin fragments by LC MS circumvents at least some of these proteins. In addition, the use of an internal standard facilitates the comparison of biological replicates in a reliable manner (286). In both the aerobic and anaerobic data sets approximately 100 integral membrane proteins containing between 1 and 13 TMSs were quantified. *E. coli* is predicted to contain 764 TMS-containing proteins (260) and in the present study 134 were quantitatively analyzed. Although this represents only 17.5% of the theoretical value, the number of proteins expressed under the conditions tested would be considerably less than 764. Also with membrane-associated, cytoplasmic, outer membrane and periplasmic proteins there was no great variation between

the aerobic and anaerobic samples in the number of proteins identified and quantified. This indicates the suitability of this method for the proteome-wide studies in *E. coli*.

The aim of this study was to identify new YidC-dependent proteins. The small metal ion transporter CorA was observed to decrease significantly under both aerobic and anaerobic conditions and was also found to co-purify with His-YidC. This 37 kDa protein has 2 TMSs with a large cytoplasmically located N-terminus. Decrease upon YidC depletion was not a general feature of helical hairpin proteins. Small hydrophobic proteins were in fact underrepresented in this study. F_oC was not identified and protein with 1 or 2 TMSs identified generally contained large C- or N-terminal tails. Protein identification tools are based on trypsin generated fragments and TMSs are largely trypsin resistant owing either to a lack of charged residues and/or protection due to folding of the hydrophobic segments. Trypsin digestion combined with chemical cleavage may allow the identification of such small hydrophobic proteins. A number of ABC transporters were found to decrease upon YidC depletion including the arginine, dipeptide, histidine, leucine-specific, lipoprotein-releasing, lipopolysaccharide and putrescine transporters. It has previously been reported that levels of the maltose transporter MalFGK decrease upon YidC depletion and that this was due to the involvement of YidC in the folding of MalF in the membrane and its incorporation into a protein complex with MalFGK (514). YidC may therefore play a role in the biogenesis of other ABC transporter subunits and/or complexes such as those identified in this study. Also the alpha-ketoglutarate permease KgtP and the proline transporter ProP decreased significantly upon YidC depletion and since these proteins share a similar topology to the lactose permease LacY, YidC may too be essential for their folding in the membrane. Not all MFS transporters were however negatively affected by YidC depletion indicating that YidC-involvement needs to be evaluated case by case. As mentioned above, YidC has been implicated in the insertion and/or oligomerization of the mechanosensitive channel MscL. Levels of the mechanosensitive ion channel MscS decreased upon YidC depletion suggesting a role for YidC in the biogenesis of this heptameric (30) ion channel. A number of putative proteins were observed to decrease upon YidC depletion. Assessment of these results is difficult since the function of these proteins is not known.

In conclusion, YidC depletion results in protein aggregation/misfolding in the cytoplasm as well as misfolding in the inner membrane of *E. coli.* The chaperone response to this stress in response to under aerobic growth conditions varied to

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that of those grown under anaerobic conditions. In addition, the present study provides a set of potential new substrates for the design of *in vitro* insertion experiments such as those already described (497,499,499) to determine the extent of YidC involvement in their biogenesis.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Netherlands Proteomics Centre (NPC). We would like to thank Axle Mogk (Universität Heidelberg, Germany), Gabriele Deckers-Hebestreit (Universität Osnabrück, Germany), Saskia van der Vies (Vrije Universiteit Amsterdam, the Netherlands), Ewa Laskowska (University of Gdansk, Poland), Axel Magalon (LCB-IBSM CNRS, Marseille, France), Takao Yagi (The Scripps Research Institute, USA) and Jan Tommassen (Utrecht University, the Netherlands) for the various antibodies.

SUPPLEMENTARY DATA

Supporting information for the identification and quantitation data presented in this study is available on request

SUPPLEMENTARY TABLE 1. Other inner membrane or inner membrane–associated prorteins identified in this study

Gene name[a]	Protein name[b]	TMS / localization[c]	Log2 (YidC+-YidC-)[d] Aerobic	Anaerobic
Proteases				
glpG	Rhomboid protease GlpG	6	-0.15	not quantified[e]
qmcA	Protein QmcA	1	0.51	not identified
lepB	Signal peptidase I	2	0.30	0.04
sohB	Probable protease SohB	1	-0.38	-0.35
sppA	Protease 4	3	0.47	-0.04
prc	Tail-specific protease	p, im ass	-0.18	-0.27
Lipoproteins				
ecnB	Entericidin B	im lp	not identified	0.16
mltC	Membrane-bound lytic murein transglycosylase C	im lp	-0.44	not identified
nlpA	Lipoprotein 28	im lp	0.38	0.33
rlpA	Rare lipoprotein A	im lp	-0.21	-0.06
yajG	uncharacterized lipoprotein YajG	im lp	0.13	-0.06
yajI	uncharacterized lipoprotein YajI	im lp	not identified	-0.08
ydcL	uncharacterized lipoprotein YdcL	im lp	-0.55	0.18
yeaY	uncharacterized lipoprotein YeaY	im lp	not identified	-0.15
yedD	uncharacterized lipoprotein YedD	im lp	not quantified	-0.10
yfhM	Uncharacterized lipoprotein YfhM	im lp	-0.12	0.26
Sensor proteins				
arcB	Aerobic respiration control sensor protein ArcB	2	-0.13	not quantified
dipZ	Thiol:disulfide interchange protein DsbD	8	-0.10	not quantified
phoQ	Sensor protein PhoQ	2	0.47	0.39
rstB	Sensor protein RstB	2	0.20	0.21
Ion channels				
clcA	H(+)/Cl(-) exchange transporter ClcA	10	0.20	-0.08
mscS	Small-conductance mechanosensitive channel	3	-0.54	not quantified
ybdG	Uncharacterized protein YbdG	6	0.06	not identified
yjeP	Uncharacterized mscS family protein YjeP	10	-0.52	-0.28
kefA	Potassium efflux system KefA	11	-0.20	-0.08
Metabolism				
gcd	Quinone glucose dehydrogenase	5	-0.46	not identified
lpdA	Dihydrolipoyl dehydrogenase	c, im ass	-0.34	0.04

mdoB	Phosphoglycerol transferase I	4	0.53	-0.32
pyrD	Dihydro-orotate dehydrogenase	c, im ass	0.46	-0.02
PTS system				
manX	PTS system mannose-specific EIIA/B component	c, im ass	0.34	-0.15
manZ	Mannose permease IID component	1	0.09	-0.10
mtlA	PTS system mannitol-specific EIICBA component	6	-0.20	0.16
nagE	PTS system N-acetyl glucosamine-specific EIICBA component	12	**0.58**	0.23
ptsG	PTS system glucose-specific EIICB component	10	-0.41	-0.33
ulaA	Ascorbate-specific permease IIC component ulaA	10	0.08	0.30
Miscellaneous				
aas	Bifunctional protein aas	2	-0.22	0.34
cdh	CDP-diacylglycerol pyrophosphatase	1	0.06	not identified
cls	Cardiolipin synthase	2	-0.25	-0.16
dedD	Protein DedD	1	-0.10	not identified
murG	Undecaprenyl-PP-MurNAc-pentapeptide-UDPGlcNAc transferase	c, im ass	0.07	not identified
plsB	Glycerol-3-phosphate acyltransferase	c, im ass	0.27	0.12
pntA	NAD(P) transhydrogenase subunit alpha	4	0.15	0.17
pntB	NAD(P) transhydrogenase subunit beta	9	0.08	0.24
ppiD	Peptidyl-prolyl cis-trans isomerase D	1	0.33	0.14
pqiB	Paraquat-inducible protein B	1	-0.34	0.43
psd	Phosphatidylserine decarboxylase proenzyme	c, im ass	-0.08	0.20
pssA	CDP-diacylglycerol-serine O-phosphatidyltransferase	c, im ass	not identified	0.06
smp	Protein Smp	p, im ass	-0.22	not quantified
ubiB	Probable ubiquinone biosynthesis protein ubiB	2	-0.24	not identified
waaA	3-deoxy-D-manno-octulosonic-acid transferase	1	0.06	-0.18
wzzB	Chain length determinant protein	2	0.07	0.14
wzzE	Lipopolysaccharide biosynthesis protein WzzE	2	0.02	0.08
yccF	Inner membrane protien YccF	3	not quantified	0.51
ydgA	Protein YdgA	p, im ass	0.12	0.39
yebO	Uncharacterized protein YebO	1	-0.11	not identified
yebT	Uncharacterized protein YebT	1	-0.13	not identified
yedE	UPF0394 inner membrane protein YedE	8	not quantified	-0.36
yfgM	UPF0070 protein YfgM	1	0.10	0.22
ygiM	Uncharacterized protein YgiM	1	0.19	0.05

SUPPLEMENTARY TABLE 1. continued

yhcB	Putative cytochrome d ubiquinol oxidase subunit 3*	1	0.12	0.06
yhjK	Protein yhjK	2	0.01	not identified
yijP	UPF0141 membrane protein YijP	5	-0.04	0.09
yniB	Uncharacterized protein YniB	3	not identified	-0.34
yrfF	Putative membrane protein igaA homolog	5	-0.39	not identified
ytfL	UPF0053 inner membrane protein YtfL	5	-0.03	not identified

[a,b] Gene and protein designations in the UniProtKB/Swiss-Prot database for *E. coli*.

[c] Localization based on Uniprot entry or where necessary Psort 2.0b. Abbreviations: c, cytoplasm; p, periplasm; im ass, inner membrane associated; im lp, inner membrane lipoprotein.

[d] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains.

[e] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.

* Annotated in the UniProtKB database as associating with the cytochrome *d* ubiquinol oxidase based on observations in (449) but this has since been shown not to be the case (314).

Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

SUPPLEMENTARY TABLE 2. Outer membrane proteins identified in this study.

Gene name[a]	Protein name[b]	Log2 (YidC$^+$-YidC$^-$)[c]	
		Anaerobic	Aerobic
amiD	N-acetylmuramoyl-L-alanine amidase AmiD	not identified	-0.01
bamA (yaeT)	Outer membrane protein assembly factor YaeT	-0.38	-0.13
bamB (yfgL)	Lipoprotein YfgL	-0.16	-0.12
bamC	Lipoprotein	0.05	-0.18
bamD	Predicted lipoprotein	-0.21	-0.16
blc	Outer membrane lipoprotein Blc	not identified	0.56
btuB	Vitamin B12 transporter BtuB	-0.21	0.13
cirA	Colicin I receptor	-0.40	not identified
emtA	Endo-type membrane-bound lytic murein transglycosylase A	**-0.80**	0.12
fadL	Long-chain fatty acid transport protein	0.35	not quantified[d]
fepA	Ferrienterobactin receptor	-0.53	-0.19
fhuE	FhuE receptor	**-0.77**	not identified
fiu	Catecholate siderophore receptor fiu	**-0.62**	not identified
imp	Lipopolysaccharide assembly protein	0.06	-0.12
lamB (malB)	Maltoporin	0.41	0.25
lolB	Outer-membrane lipoprotein LolB	0.07	0.09
lpp	Major outer membrane lipoprotein	-0.10	0.29
lptE	LPS-assembly lipoprotein LptE	not quantified	0.01
mltA	Membrane-bound lytic murein transglycosylase A	not quantified	0.03
nlpD	Lipoprotein NlpD	**-1.3**	not quantified
nlpE	Lipoprotein NlpE	not quantified	0.08
ompA	Outer membrane protein A	0.47	0.15
ompC	Outer membrane protein C	**-1.05**	**-0.72**
ompF	Outer membrane protein F	0.45	-0.03
ompT	Protease 7	-0.01	0.32
ompW	Outer membrane protein W	not identified	0.38
ompX	Outer membrane protein X	0.20	-0.21
pal	Peptidoglycan-associated lipoprotein	-0.20	0.09
pldA	Phospholipase A1	not identified	0.10
rcsF	Protein rcsF	not identified	0.14
rfaF	ADP-heptose--LPS heptosyltransferase 2	not identified	0.35
slp	Outer membrane protein slp	not identified	**0.76**
slyB	Outer membrane lipoprotein slyB	0.50	0.18
tolC	Outer membrane protein TolC	0.33	0.04
tsx	Nucleoside-specific channel-forming protein Tsx	0.36	0.05
uidC	Membrane-associated protein uidC	not identified	0.07
vacJ	Lipoprotein vacJ	0.11	-0.03
ybaY	Hypothetical lipoprotein ybaY	not quantified	0.01
ybhC	Putative acyl-CoA thioester hydrolase ybhC	0.45	0.15
ybjP	Uncharacterized lipoprotein ybjP	not identified	0.12
ycfM	Uncharacterized protein ycfM	-0.59	0.00
yddB	Uncharacterized protein yddB	-0.25	not identified
ygaU	Uncharacterized protein ygaU	not identified	0.25
ygiB	UPF0441 protein ygiB	**-1.51**	-0.25
yhhM	Uncharacterized protein yhhM	not quantified	0.12
yjaH	Uncharacterized protein yjaH	not identified	0.12
yraM	Uncharacterized protein yraM	-0.58	-0.28

[a,b] Gene and protein designations in the UniProtKB/Swiss-Prot database for *E. coli*.

[c] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between YidC$^+$ and YidC$^-$ strains.

[d] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.

Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

SUPPLEMENTARY TABLE 3. Periplasmic proteins identified in this study

Gene name[a]	Protein name[b]	Log2 (YidC$^+$-YidC$^-$)[c]	
		Anaerobic	Aerobic
agp	Glucose-1-phosphatase	not identified	-0.15
ansB	L-asparaginase 2	not identified	**-1.08**
asmA	Protein asmA	0.19	0.17
dacB	D-alanyl-D-alanine carboxypeptidase DacB	**-0.82**	not identified
dcrB	Protein DcrB	-0.07	0.15
degP	Protease do	-0.49	**-1.13**
degQ	Protease DegQ	not identified	**-1.32**
degS	Protease DegS	-0.29	not quantified[d]
fkpA	FKBP-type peptidyl-polyl cis-trans isomerase FkpA	not quantified	-0.36
fliY	Cystine-binding periplasmic protein	-0.41	0.01
glpQ	Glycerophosphoryl diester phosphodiesterase	not identified	-0.58
mglB	D-galactose-binding periplasmic protein	not identified	-0.51
oppA	D-galactose-binding periplasmic protein	**-1.29**	-0.12
skp	Chaperone protein Skp	**-0.76**	0.20
surA	Chaperone SurA	**-1.09**	-0.49
tolB	Protein TolB	-0.21	-0.07
ybiS	Uncharacterized protein YbiS	not quantified	-0.06
yhjA	Uncharacterized protein YhjA	not identified	0.28
yraP	Uncharacterized protein YraP	-0.18	0.07

[a,b] Gene and protein designations in the UniProtKB/Swiss-Prot database for *E. coli*.
[c] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between YidC$^+$ and YidC$^-$ strains.
[d] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.
Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

SUPPLEMENTARY TABLE 4. Proteins containing a signal sequence but to which no localization could be predicted

Gene name[a]	Protein name[b]	Log2 (YidC$^+$-YidC$^-$)[c]	
		Anaerobic	Aerobic
yhiL	Putative uncharacterized protein YhiL	not quantified[d]	**0.74**
anmK	Anhydro-N-acetylmuramic acid kinase	**-1.78**	-0.19
ybgF	Uncharacterized protein YbgF	not quantified	-0.40
ydjY	Uncharacterized protein YdjY	not quantified	0.22
yncE	Uncharacterized protein YncE	**-0.99**	not identified
yoaE	Probable ATP-dependent helicase YoaA	-0.05	not identified

[a,b] Gene and protein designations in the UniProtKB/Swiss-Prot database for *E. coli*.
[c] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between YidC$^+$ and YidC$^-$ strains.
[d] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.
Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.

SUPPLEMENTARY TABLE 5. Cytoplasmic proteins exhibiting significant changes upon YidC depletion.

Gene name[a]	Protein name[b]	Log2 ($YidC^+$-$YidC^-$)[c]	
		Anaerobic	Aerobic
adhE	Aldehyde-alcohol dehydrogenase	-1.66	not identified
citE	Citrate lyase beta chain	not identified	1.01
citF	Citrate lyase alpha chain	not identified	0.71
deaD	Cold-shock DEAD box protein A	2.10	not identified
dps	DNA protection during starvation protein	not identified	0.68
fusA	Elongation factor G (Ef-G)	1.11	not identified
metF	5,10-methylenetetrahydrofolate reductase	1.02	not identified
mqo	Malate:quinone oxidoreductase	-1.26	not identified
yjiL	Uncharacterized protein yjiL	not identified	0.68
yqjD	Uncharacterized protein yqjD	not identified	0.60

[a,b] Gene and protein designations in the UniProtKB/Swiss-Prot database for *E. coli*.
[c] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains.

SUPPLEMENTARY TABLE 6. Ribosomal proteins

Gene name[a]	Protein name[b]	Log2 ($YidC^+$-$YidC^-$)[c]	
		Anaerobic	Aerobic
rpsB	30S ribosomal subunit protein S2	0.16	0.15
rpsA	30S ribosomal subunit protein S1	0.25	-0.14
rplT	50S ribosomal subunit protein L20	0.39	0.14
rpsI	30S ribosomal subunit protein S9	0.34	-0.11
rplM	50S ribosomal subunit protein L13	not identified	0.35
rplQ	50S ribosomal subunit protein L17	0.32	0.17
rpsD	30S ribosomal subunit protein S4	0.35	-0.05
rpsK	30S ribosomal subunit protein S11	not quantified[d]	-0.11
rplO	50S ribosomal subunit protein L15	0.28	-0.33
rpsE	30S ribosomal subunit protein S5	0.46	0.01
rplR	50S ribosomal subunit protein L18	not quantified	-0.10
rplF	50S ribosomal subunit protein L6	0.36	-0.22
rpsH	30S ribosomal subunit protein S8	not quantified	0.01
rplE	50S ribosomal subunit protein L5	0.04	0.05
rplN	50S ribosomal subunit protein L14	not quantified	0.22
rplP	50S ribosomal subunit protein L16	0.26	0.21
rpsC	30S ribosomal subunit protein S3	0.23	0.08
rplV	50S ribosomal subunit protein L22	not quantified	0.24
rplB	50S ribosomal subunit protein L2	0.12	-0.14
rplD	50S ribosomal subunit protein L4	not identified	-0.13
rpsG	30S ribosomal subunit protein S7	0.44	-0.06
rpmB	50S ribosomeal subunit protein L28	not identified	0.14
rplK	50S ribosomal subunit protein L11	**0.58**	-0.05
rplA	50S ribosomal subunit protein L1	0.44	not quantified
rplJ	50S ribosomal subunit protein L10	0.46	-0.01
rplL	50S ribosomal subunit protein L7/L12	not identified	-0.15
rpsF	50S ribosomal subunit protein L9	**0.62**	-0.08
rplI	50S ribosomal subunit protein L9	0.38	-0.02

[a,b] Gene and protein designations in the UniProtKB/Swiss-Prot database for *E. coli*.
[c] The $\log_2$ ratio of each sample to the ^{15}N reference was used to calculate the difference in protein levels between $YidC^+$ and $YidC^-$ strains
[d] Not quantified: Protein was identified in at least one sample but insufficient data for quantitation.
Numbers in bold indicate a change of 1.5-fold or more upon YidC depletion.